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ORIGINAL ARTICLE

A lipidomic analysis approach to evaluate the response to cholesterol-lowering food intake

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Abstract Plant sterols (PS) are well known to reduce serum levels of total cholesterol and LDL-cholesterol. Lipidomics potentially provides detailed information on a wide range of individual serum lipid metabolites, which may further add to our understanding of the biological effects of PS. In this study, lipidomics analysis was applied to serum samples from a placebo-controlled, parallel human intervention study ($n = 97$) of 4-week consumption of two PS-enriched, yoghurt drinks differing in fat content (based on 0.1% vs. 1.5% dairy fat). A comprehensive data analysis

strategy was developed and implemented to assess and compare effects of two different PS-treatments and placebo treatment. The combination of univariate and multivariate data analysis approaches allowed to show significant effects of PS intake on the serum lipidome, and helped to distinguish them from fat content and non-specific effects. The PS-enriched 0.1% dairy fat yoghurt drink had a stronger impact on the lipidome than the 1.5% dairy fat yoghurt drink, despite similar LDL-cholesterol lowering effects. The PS-enriched 0.1% dairy fat yoghurt drink reduced levels of several sphingomyelins which correlated well with the reduction in LDL-cholesterol and can be explained by co-localization of sphingomyelins and cholesterol on the surface of LDL lipoprotein. Statistically significant reductions in serum levels of two lysophosphatidylcholines (LPC(16:1), LPC(20:1)) and cholesteryl arachidonate may suggest reduced inflammation and atherogenic potential.

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1 Introduction

Much is expected from nutritional metabolomics. It should provide a powerful approach to better identify and understand the often subtle effects of dietary ingredients on human metabolism and health (Hunter 2009). Advances in nutritional metabolomics allow a more extensive analysis of the individual response to a nutritional intervention and a reliable assessment of the contribution of specific metabolites and metabolic pathways to physiological processes related to health and disease (Orešič 2009a; Lodge 2010; Müller and Kersten 2003).

It is known that in human intervention studies the inter-individual variability in body fluid metabolic profiles can easily dominate over the average response to the nutritional intervention (Brennan 2008; Walsh et al. 2006). Therefore, to draw meaningful conclusions, well defined research questions, suitable experimental design, high quality of metabolomic data and powerful data analysis tools are essential in all nutritional metabolomics studies.

Elevated plasma concentrations of total cholesterol (TC) and LDL-cholesterol (LDL-C) are associated with increased risk for coronary heart disease in humans. While statins (or HMG-CoA reductase inhibitors) have been the major treatment option for hypercholesterolemic patients, nutritional approaches including plant sterols (PS) enriched food products have become available for treatment and prevention of milder forms of hypercholesterolemia (Demonty et al. 2009; Katan et al. 2003). Plant sterols are naturally occurring phytochemicals, which are structurally related to cholesterol and their cholesterol-lowering properties have been ascribed to reduced absorption of cholesterol from the gastrointestinal tract (de Jong et al. 2003; Trautwein et al. 2003). The cholesterol-lowering efficacy of PS intake is well-known, but it is not clear whether this also leads to metabolic effects at the level of specific plasma lipids, which are in dynamic exchange between lipoproteins and peripheral membranes. Lipidomics has recently been applied to study lipoprotein metabolism in both health and disease states (Ekroos et al. 2010; Kontush and Chapman 2010; Orešič 2009a, b) e.g. to evaluate (adverse) effects of lipid-modifying drug treatments like statins (Bergheanu et al. 2008; Kaddurah-Daouk et al. 2010; Laaksonen et al. 2006). Lipoproteins are known to differ in lipid composition, but it remains to be established whether this is relevant for lipoprotein function, and also whether changes in lipoprotein lipids provide stronger risk prediction (Hidaka et al. 2007; Sysi-Aho et al. 2007; Wiesner et al. 2009; Yetukuri et al. 2010).

A recent placebo-controlled, parallel group study by Doornbos et al. (2005) demonstrated that two PS-enriched single-dose yoghurt drinks were able to effectively lower TC and LDL-C concentrations irrespective of the fat content of the drink. The main objective of our study was to expand observations of Doornbos et al. and determine whether and how those PS-enriched yoghurt drinks affect serum lipidomics profiles. The lipidomics data set was used to gain new insights into lipid biochemical changes occurring during an intervention related to PS effects, fat content effects and non-specific effects of tested yoghurt drinks. Application of a new comprehensive data analysis strategy taking into account a parallel study design, complex study aim and minor alterations in the serum lipidome distributed across multiple lipid metabolites expected, was an important step of our study. Finally, found nutritional

effects were related to changes in lipoprotein compositions observed by Doornbos et al. to explore and understand associations between changes in serum lipoprotein and lipidomic profiles.

2 Materials and methods

2.1 Ethics Statement

The original design and aims of this study have been described in detail by Doornbos et al. (2005). The study was performed and conducted at Unilever R&D Vlaardingen, The Netherlands in 2004/2005, following the guidelines of Good Clinical Practice (ICH-GCP). Participants were informed about the study both verbally and written and all subjects signed an informed consent form before participation. The study was approved by the Medical Ethical Committee of Wageningen University (February 2004).

2.2 Study design

The primary aim of the human intervention study, which was published by Doornbos et al. (2005), was to determine the LDL-C lowering efficacy of PS-enriched yoghurt drinks and examine the effects of fat content and intake occasion. The study was a randomized, double-blind, placebo-controlled, parallel intervention study with five treatment arms. The current study used serum samples that had been stored at -80°C and examined how serum lipidomics can provide additional information on lipid metabolism supporting the LDL-C lowering effects of PS intake. In the original study the subjects were randomly allocated to one of five different treatments (Doornbos et al. 2005) but only three of these treatments are included in this metabolomic study. After a run-in period of 4 weeks, during which the subjects were encouraged to minimize changes in composition of their habitual diet and TC was measured, volunteers were asked to daily consume one of three 100-g single-dose yoghurt drinks with/without immediately after lunch, during a period of 4 weeks. Drink A was a yoghurt drink containing 0.1% dairy fat and 2.2% total fat plus 3.2 g PS, drink B was a yoghurt drink with 1.5% dairy fat and 3.3% total fat plus 2.8 g PS and drink C was a placebo yoghurt drink (based on 1.5% total fat which was 1.5% dairy fat) without added PS. All drinks had similar content of carbohydrates and proteins. For a more detailed nutritional composition please see Doornbos et al. (2005).

Serum samples from each volunteer were collected at the end of the run-in period (baseline sample, before intervention sample) and at the end of the 4-week treatment period (after intervention sample). Serum samples for the

lipidomics study had been stored since collection at -80°C . Before blood sampling, subjects were fasted and had refrained from food for at least 10 h. Venous blood samples (5 ml) were taken into plain tubes. Blood samples were centrifuged for 10 min at 3,000 r.p.m. at 22°C to separate serum from red blood cells and serum was stored at -70°C until analysed. Standard serum lipoprotein profiles (TC, LDL-C, HDL-C and triacylglycerol (TAG)) were measured on a Hitachi 912 Autoanalyser (Roche Diagnostics, Basel, Switzerland), as described in Doornboos et al. (2005).

2.3 Study participants

Subjects were selected based on screening values for mean fasting TC between 5.0 and 8.0 mmol/l, mean fasting TAG 4.0 mmol/l, body mass index (BMI) between 18.0 kg/m^2 and 32.0 kg/m^2 , no use of lipid lowering medication or medication potentially affecting the study measurements, supplements or a diet known to affect serum lipids. The baseline characteristics of the study participants are shown in Table 1. There were no significant differences in baseline characteristics between the groups.

2.4 Analytical methods

The UPLC-MS Lipidome Platform was developed, validated and applied in this study at the Demonstration and Competence Laboratory, Netherlands Metabolomics Centre at Leiden University, Leiden, The Netherlands.

From each serum sample, duplicate 30 μl aliquots were taken for lipidomic analysis. For preparation of quality control samples (QC) a pooled sample consisting of equal amounts of plasma from all study samples was used. Blank samples contained 30 μl of HPLC-MS grade water. Study samples, quality control samples and blank samples underwent the same sample preparation procedure. To a 30 μl serum aliquot a mixture of internal standards and calibration standards (Supplementary Table 1) was added

followed by liquid–liquid extraction (LLE) with a (2:1) mixture of dichloromethane and methanol according to the procedure described in detail in Hu et al. (2008). The organic phase containing most of the lipids was taken for further analysis.

The Acquity UPLC system (Waters, Milford, MA, USA) and Agilent 6,530 Accurate mass Q-TOF LC/MS (Agilent Technologies, Waldbronn, Germany) were used in this study. An Ascentis Express C8 $2.1 \times 150\text{ mm}$ (2.7 μm particle size) column was used as a separation column. Experimental conditions during chromatographic analysis and detection by mass spectrometry were similar to the protocol described by Hu et al. (2008) with only minor modification in UPLC solvent gradient. A binary gradient system of acetonitrile–water (60:40, v:v) (eluent A) and isopropanol–acetonitrile (90:10, v:v) (eluent B), both containing 10 mM ammonium formate was used containing the following gradient steps in a total analysis time of 20 min: 0–1 min isocratic elution with 32% B, 1–3 min increase to 50% B, 3–9 min increase to 65% B, 9–14 min increase to 80% B, 14–14.1 min increase to 97% B and 14.1–17 min maintenance of 97% B, 17–17.1 decrease to 32% B and 17.1–20 min isocratic elution with 32% B. The flow rate was 0.5 ml/min.

The applied UPLC-MS Lipidomics Platform allowed the identification of more than 100 lipid metabolites on the basis of retention time and mass-to-charge ratio (m/z). A complete list of the 106 metabolites detected in the studied samples is presented in the Supplementary Table 2. Lipid nomenclature according to Lipids Maps database (<http://www.lipidmaps.org/>) was used. After exclusion of five metabolites during quality control profiles of 101 lipid metabolites were obtained. They were composed of metabolites from 8 different lipid subclasses: three cholesteryl esters (CEs), one diacylglycerol (DG), 15 lysophosphatidylcholines (LPCs), one lysophosphatidylethanolamine (LPE), 31 phosphatidylcholines (PCs), six phosphatidylethanolamines (PEs), 20 sphingomyelins (SMs) and 23 triacylglycerols (TGs).

Table 1 Baseline characteristics of study participants including mean values \pm standard deviation for 6 parameters: age, BMI, HDL-C, LDL-C, TC and TAG

Characteristics	All ($n = 97$)	Group A ^a ($n = 33$)	Group B ^b ($n = 35$)	Group C ^c ($n = 29$)
Age (years)	57.86 ± 11.35	56.49 ± 15.22	58.69 ± 9.01	58.41 ± 8.65
BMI (kg/m^2)	25.58 ± 3.02	25.01 ± 2.94	25.65 ± 3.16	26.13 ± 2.92
HDL-C (mmol/l)	1.63 ± 0.47	1.75 ± 0.50	1.58 ± 0.43	1.53 ± 0.48
LDL-C (mmol/l)	4.01 ± 0.79	4.10 ± 0.96	3.81 ± 0.71	4.15 ± 0.67
TC (mmol/l)	6.14 ± 0.82	6.31 ± 0.92	5.90 ± 0.78	6.22 ± 0.69
TAG (mmol/l)	1.47 ± 0.73	1.40 ± 0.64	1.48 ± 0.85	1.57 ± 0.70

^a Group of subjects treated with plant sterol (PS)-enriched yoghurt drink with 0.1% dairy fat and 2.2% total fat

^b Group of subjects treated with PS-enriched yoghurt drink with 1.5% dairy fat and 3.3% total fat

^c Group of subjects treated with placebo yoghurt drink with 1.5% dairy fat

Recently, Psychogios and Hau (2011) reported on the analysis of *ca.* 3,400 lipids in human serum. Although these numbers are impressive, they are mainly originating from estimation of presence of individual phospholipids by using CLR, a combinatorial approach to estimate the lipids presence of a specific lipid on the basis of the fractional abundance of each fatty acid chain and the total concentration of a given lipid class. The method employed in this paper however, is based on the actual presence of target compounds in a LC-MS raw data file. The target compounds are previously identified by LC-high resolution MS, using the FT-ICR-MS, and (relative) retention time as reported by Hu et al. (2008). Therefore, reporting the presence of a compound has a high degree of certainty in this approach.

Lipid metabolites were quantified using Agilent Quantitative Analysis software Version B01.04 Build 1.4.126.2 and Version B03.01 Build 3.1.170.0 (Agilent Technologies, Waldbronn, Germany). Peak areas of the studied lipids were normalized to that of an appropriate lipid internal standard. The internal standards and calibration standards used are representatives of the 5 major subclasses of phospholipids measured. As internal standards and calibration standards, phospholipids with an odd nr. of carbon atoms (saturated C17 and C15¹ fatty acyl groups in case of the internal standards and the calibration standards, respectively) are used due to their extremely low presence/absence in human plasma. Phospholipids having unsaturated fatty acyl chains are in this study assumed to have identical response factors as their corresponding phospholipids with saturated fatty acyl chains. In summary, the LPCs are corrected by an LPC; the PCs, SMs and DGs are corrected by a PC; the LPEs and PEs are corrected by a PE; and finally the TGs and the CEs are corrected for by a TG internal standard. Plasmalogens (ether lipids, labeled with symbol O-) in the various groups viz. LPCs, PCs, LPEs and PEs are corrected for by the identical internal standard as used for their corresponding phospholipids. DGs, CEs and SMs are corrected by an internal standard belonging to another group of phospholipids. During validation and application of the method, the listed internal standards do correct adequately for drift observed for individual compounds during the analysis of a particular batch as described by Hu et al. (2008). Calibration standards are used to check for consistent linearity and sensitivity of the method. Measurement design and quality control issues are presented in Supplementary Material.

¹ In the case of phosphatidylglycerol (PG), a C14 acyl chain phospholipid was used as calibration standard and in the case of LPC and PC a C19 acyl chain phospholipid was used as calibration standard.

2.5 Data set structure

The data set structure after data acquisition and quality control was as follows: 97 subjects \times 101 lipid metabolites \times 2 time points: before (baseline) and after treatment period \times 2 real sample replicates (new sample preparation and UPLC-MS analysis). Mean values of metabolites levels were taken across two real sample replicates giving the data set structure of 97 subjects \times 101 lipid metabolites \times 2 time points (Supplementary Fig. 1, panel D0) and this data set after preprocessing was used in data analysis comprising three different approaches.

2.6 Data analysis strategy

It can be anticipated that if data structure and underlying research questions are taken into account, the data analysis becomes more focused on relevant sources of variation and therefore has more power (Szymańska et al. 2007; Thissen et al. 2009). In case of our study three factors should be included in the development of the data analysis strategy (as shown in Fig. 1): the characteristics of the lipidomics data set, the parallel study design and the complex aim of the study comprising analysis of plant sterol effects, fat content effects and non-specific effects. The study design and complex aim call for different ways of statistical analysis that allow to compare not only effects of the two PS-enriched drinks and the placebo drink (plant sterol and fat content effects), but also to compare effects of each intervention with respect to the baseline (to distinguish between specific and non-specific effects). That requires in total 6 different models: (1) effect of PS-enriched drink A versus effect of placebo drink C, (2) effect of PS-enriched yoghurt B versus effect of placebo drink C (both to assess plant sterol effects), (3) effect of PS-enriched drink A versus effect of PS-enriched drink B (to assess fat content effect), (4–6) before versus after treatment with drink A, B and C (to distinguish specific and non-specific effects). A two-level evaluation of the observed nutritional effects was an important part of developed strategy and included assessment of post-intervention effects on the lipidome with respect to the baseline lipidome, as well as pairwise comparison of the effects of the different drinks. The data analysis strategy comprised multivariate as well as univariate analysis to account for changes in lipidome distributed across multiple lipid metabolites, and pairing of data from the same individual to correct for inter-individual variation in baseline lipid levels. In practice, the applied data analysis strategy consisted of three data analysis approaches comprising one univariate (first data analysis approach) and two complementary multivariate data analysis approaches (second and third data analysis approaches).

In the first data analysis approach, linear mixed models for repeated measurements were used. This method is not

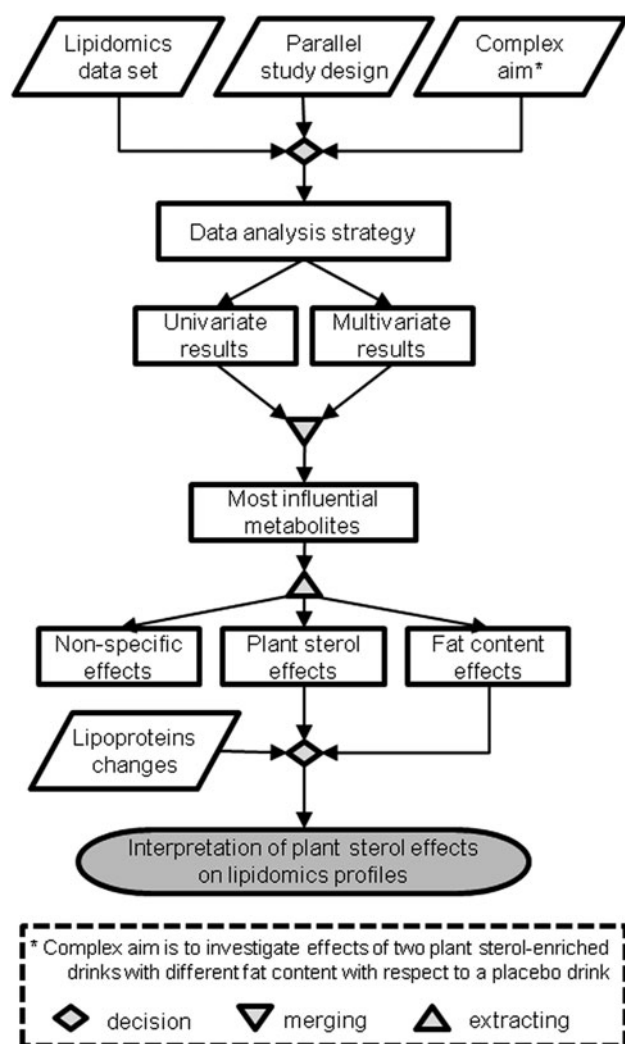


Fig. 1 Flowchart of this study

commonly used in metabolomics (Schwab et al. 2008) but it is routinely used in human nutritional and clinical trials because it can implement study design information and known covariates into the model (Rajpathak et al., in press; Romaguera et al. 2010). By using the repeated observations from the same individual, inter-individual variation could be estimated (Fig. 2a) and mean effects for each lipid metabolite for all three interventions could be simultaneously assessed and compared (Fig. 2b). The net effect of each treatment (δ) was studied as the interaction of intervention time and treatment group (Fig. 2b) and effects of different treatments could be simultaneously estimated and compared by statistical testing with *F*-test.

The drawback of linear mixed models is that they consider all lipids separately and thus ignore the complex correlation structure of lipidomics data (Orešič 2009a). Studying multivariate changes in metabolite profiles may be particularly relevant for lipidomics, since % concentration changes of lipids across different phenotypes are

usually small and distributed across multiple lipid species (Orešič 2009b; Yetukuri et al. 2007). Therefore, two multivariate data analysis approaches were also included in our data analysis strategy. Multilevel-PLS-DA (M-PLS-DA) analysis was included to study the multivariate effects of each intervention versus baseline (second approach), and also to compare multivariate effects between the different treatments (third approach). Multilevel data analysis has been already used to separate between-subject variation from within-subject variation in studies with a double crossover design (van Dorsten et al. 2006; van Velzen et al. 2008). A crossover study design where each subject participates in both a control and test intervention has the advantage that it allows for a within-subject (or intra-subject) comparison between treatments (each subject serves as its own control) by removing the between-subject (or inter-individual) variability from the comparison (Chow et al. 2007; Lodge 2010). However, in the case of our parallel design study with three treatment groups such a single analysis was not possible. Instead, six different M-PLS-DA analyses were performed including three comparisons by the second approach and three comparisons by the third approach.

The second data analysis approach involved investigation of multivariate changes with respect to baseline for each treatment group (intervention time related changes). For each treatment group (A, B and C) separately lipidomics profiles before and after the intervention period were compared by PCA and M-PLS-DA (Fig. 2c).

The third data analysis approach involved investigation of multivariate time \times treatment interactions in pairwise comparisons of three treatments effects by PCA and M-PLS-DA. In this approach, the difference (δ) between levels of metabolite *j* after and before treatment (across time) was calculated per subject *i* (Fig. 2d) (Rubingh et al. 2011).

2.7 Data analysis methods

All statistical analyses besides linear mixed models were performed using Matlab (version 2010a, The MathWorks, Natick, Massachusetts, USA). Linear mixed models (LMM) were obtained using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA).

Linear mixed models were fitted applying the method of residual maximum likelihood (REML) and implementing a compound symmetry structure on the covariances of residuals assuming dependence of residuals from the same individuals. The outcome of the linear mixed model for each metabolite was a *P*-value of an *F* test with the H_0 hypothesis that there is no change in time \times treatment interaction between the three treatments. If this *P*-value was lower than 0.05 (statistically significant) then separate

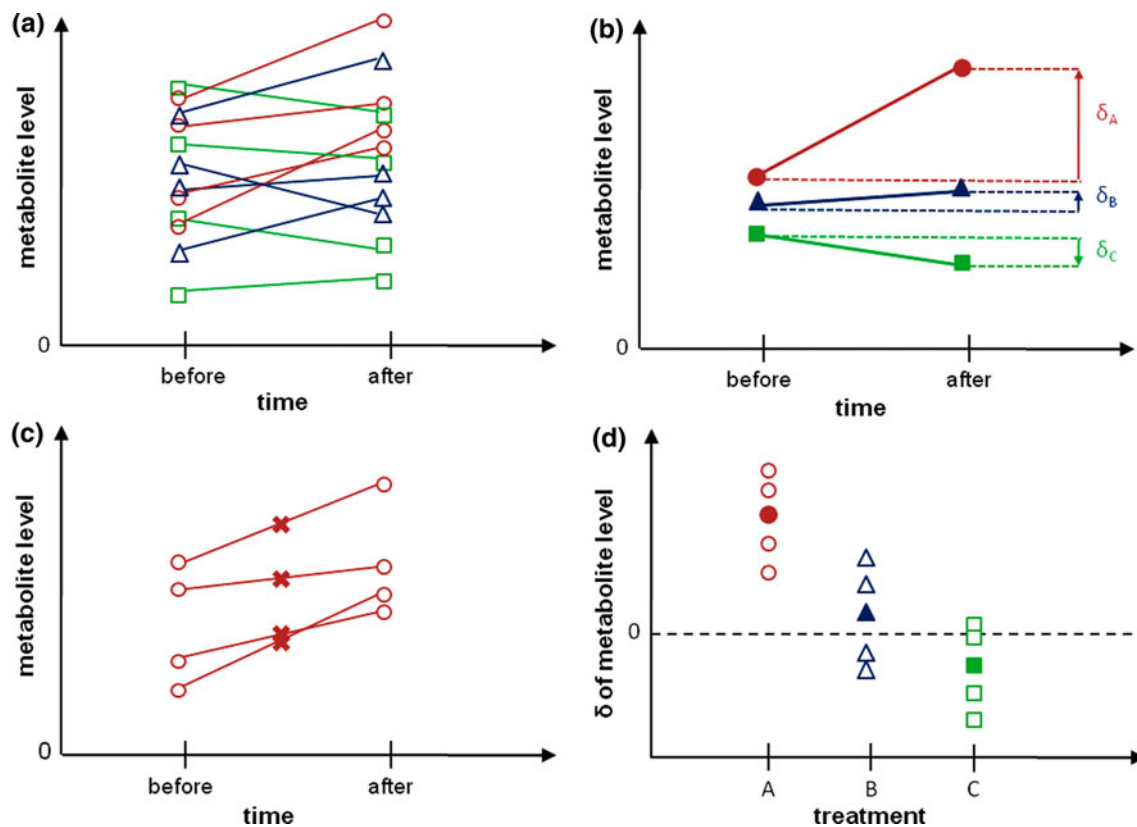


Fig. 2 Schematic representation of different aspects of analysis on effects of 3 different treatments: A (circle), B (triangle) and C (square) on levels of single metabolites. **a** individual metabolite levels (open circles, open triangles and open squares) before and after three different treatments, **b** mean levels of metabolite (full circles, full triangles and full squares) before and after three different treatments and effects of

t-tests for six tested hypotheses (six different comparisons) were performed and mean differences were estimated. *P*-values of the latter *t*-tests were corrected for multiple comparisons with a False Discovery Rate (FDR) with a significance threshold $q < 0.05$. Mean differences were expressed in % compared to baseline levels.

Principal component analysis (PCA) was performed to visualize the variation of lipidomic profiles before and after treatment period for each of treatment group (second data analysis approach—comparisons A_{before^*} versus A_{after^*} , B_{before^*} versus B_{after^*} , C_{before^*} versus C_{after^*} (Supplementary Fig. 1, panel D2)). PCA was also used to visualize the variation in treatment effects among groups A, B and C on the basis of differences in lipidomic profiles (third data analysis approach—comparisons $A_{\text{difference}}$ versus $C_{\text{difference}}$, $B_{\text{difference}}$ versus $C_{\text{difference}}$ and $A_{\text{difference}}$ versus $B_{\text{difference}}$ (Supplementary Fig. 1, panel D3)). In all cases data were autoscaled before PCA analysis.

M-PLS-DA analyses were applied to analyze multivariate effects of the three different interventions (A, B, C). In the second data analysis approach three M-PLS-DA models, namely models A_{before^*} versus A_{after^*} , B_{before^*} versus

each treatment (δ) expressed as difference in metabolite levels before and after treatment period, **c** individual metabolite levels before and after single treatment (e.g. treatment A, open circles) with mean levels for each individual (crosses), **d** individual (open circles, open triangles and open squares) and mean (full circle, full triangle, full square) differences in levels before and after each of three treatments

B_{after^*} , C_{before^*} versus C_{after^*} were built (van Velzen et al. 2008). Metabolite profiles which belong to subjects from treatment group A, B or C, respectively (as presented in Supplementary Fig. 1, panel D2) were autoscaled and used in M-PLS-DA analysis. There, metabolic profiles were discriminated by the time point label (−1 before treatment period, 1 after treatment period). In the third data analysis approach three other M-PLS-DA models, namely $A_{\text{difference}}$ versus $C_{\text{difference}}$, $B_{\text{difference}}$ versus $C_{\text{difference}}$ and $A_{\text{difference}}$ versus $B_{\text{difference}}$, were built to study multivariate time \times treatment interaction. Metabolite profiles which belong to subjects from two out of three treatment groups e.g. A and C were autoscaled and used in M-PLS-DA analysis (as presented in Supplementary Fig. 1, panel D3). In M-PLS-DA metabolic profiles were discriminated by the treatment type label (e.g. −1 for treatment group A, 1 for treatment group C).

All six M-PLS-DA analyses included double cross validation and variable selection (Smit et al. 2007; van Velzen et al. 2008). A schematic representation and detailed description of the most important steps of the M-PLS-DA procedure with variable selection is presented in the

Supplementary Material. Briefly, during 9-fold single cross validation (1CV), the optimal number of latent variables and an optimal set of variables (5-step variable selection) were chosen. In a 10-fold double cross validation (2CV), the performance of the model was evaluated by the area under the receiver operating curve (AUROC). The statistical significance of the model performance and the metabolites selected during model optimization as being the most important for the model were assessed with 2,000 permutations.

Correlation analysis was done using a dedicated Matlab GUI written in-house (Unilever R&D Vlaardingen), and involved calculation of Spearman correlation coefficients, their *P*-values and graphical display of significant correlations. Differences (δ) in 101 metabolite levels before and after the intervention period (Supplementary Fig. 1, panel D3) were correlated with differences in levels of four lipoprotein profile parameters (HDL-C, LDL-C, TC and TAG), for each intervention group (A, B and C), separately.

Differences in levels of 20 SMs before and after the intervention period were regressed against differences in levels of LDL-C and TC separately for each type of treatment (A, B or C). As a regression method partial least squares (PLS) regression with double cross validation was used. The performance of the regression models was assessed by evaluation of the root mean squared error of prediction (RMSEP) and the Pearson correlation coefficient (*r*) between predicted and real values of differences in levels of LDL-C and TC.

3 Results

3.1 Intervention effects on lipidome

Univariate analysis using linear mixed models allowed detecting significant changes in levels of 49 lipid metabolites after at least one of the tested treatments, when compared to baseline or to the effect of other treatments (see Table 2).

PCA (unsupervised multivariate analysis) did not show any discrimination between serum lipidomes before and after intervention for any of three interventions (data not shown). That was expected because of the typically high inter-subject variation in relation to nutritional effect. The results of the PCA analysis in which effects of interventions were compared in pairs e.g. effects of intervention A versus effects of intervention C are presented in Supplementary Fig. 2. Scores of groups B and C showed greater overlap than scores of groups A and C which indicates that effects of the PS-enriched drink B on the serum lipidome were weaker than effects of the PS-enriched yoghurt drink A.

Moreover in supervised multivariate analysis, six statistically significant M-PLS-DA models were obtained, namely: A_{before^*} versus A_{after^*} , B_{before^*} versus B_{after^*} , C_{before^*} versus C_{after^*} , $A_{\text{difference}}$ versus $C_{\text{difference}}$, $B_{\text{difference}}$ versus $C_{\text{difference}}$ and $A_{\text{difference}}$ versus $B_{\text{difference}}$ (Supplementary Table 4). That means that all three 4-week interventions had significantly different effects on the serum lipidome with respect to baseline and with respect to each other. For each model an optimal set of metabolites with the highest relevance for discrimination was selected and all these metabolites are listed in Table 2.

It should be noticed that the M-PLS-DA model for the placebo intervention (model C_{before^*} vs. C_{after^*}) was also significant, which means that a 4-week placebo intervention period also had statistically significant effects on the lipidome. The strongest models (with the lowest *P*-value) were obtained when the multivariate effects of the 0.1% dairy fat PS-enriched drink A were compared with baseline and to the effects of the placebo drink C. The M-PLS-DA model $A_{\text{difference}}$ versus $B_{\text{difference}}$ was only a borderline significant with a *P*-value close to 0.05 so arguably the different effects of the PS-enriched drink A and the PS-enriched drink B on the serum lipidome are statistically not so strong.

Table 2 presents mean differences in individual lipid levels which are found to be statistically significant either in univariate or in multivariate data analysis. These differences are presented for each of the three interventions with respect to the baseline levels as well as with respect to other intervention effect. In total 50 out of 101 included serum lipids were affected by at least one of the three drinks. Changes in metabolite levels after the 0.1% dairy fat PS-enriched drink (drink A) intake were usually the largest while after the placebo drink (C) they were the smallest. The effects of the 1.5% dairy fat PS-enriched drink (drink B) were for most metabolites smaller than effects of the other PS-enriched drink A.

Observed intervention effects were unevenly distributed within different lipid subclasses. In the group of cholesteryl esters, all three included metabolites (CE(18:2), CE(20:4) and CE(22:6)) showed a decrease up to ca. 12% with respect to baseline levels for the PS interventions A and B. When these effects were compared to the effects of the placebo intervention, they were statistically significant only for intervention A and for metabolites CE(18:2) and CE(20:4) (ca. 7.5% decrease with respect to placebo effect). In the group of lysophosphatidylcholines, the serum concentrations of most lipid metabolites (13 out of 15 included metabolites) decreased by more than 10% (even up to 27%) with respect to baseline levels for both PS interventions. However, changes in levels of some LPCs after the placebo drink C were also prominent and statistically significant for example for LPC(O-16:0), LPC(16:0)

Table 2 Effects of three interventions on lipidome. Effects are given within each intervention group (A, B, C) with respect to baseline lipid levels as well as between effect in one intervention group and effect in the other intervention group

Lipid ^a	Effect ^b of intervention ^c versus baseline			Effect of intervention versus effect of other intervention			Assigned effect ^d
	A	B	C	A versus C	B versus C	A versus B	
CE(18:2)	-8.03 ± 1.89 (u)	-1.87 ± 1.84	-0.63 ± 2.01	-7.40 ± 2.73 (u)	-1.24 ± 2.69	-6.16 ± 2.60 (m)	PS-A
CE(20:4)	-10.61 ± 1.87 (u, m)	-5.77 ± 1.81 (u)	-3.14 ± 1.99	-7.47 ± 2.71 (u)	-2.63 ± 2.67	-4.83 ± 2.58	PS
CE(22:6)	-6.18 ± 4.22	-11.73 ± 4.10 (u)	-11.31 ± 4.49	5.13 ± 6.07	-0.43 ± 5.99	5.56 ± 5.79	
LPC(O-14:0)	-14.92 ± 4.93 (u)	-13.14 ± 4.80	-2.31 ± 5.24	-12.61 ± 6.98	-10.83 ± 6.89	-1.78 ± 6.65 (m)	
LPC(O-16:1)	-19.06 ± 2.91 (u, m)	-10.85 ± 2.83 (u)	-8.15 ± 3.09	-10.90 ± 4.12 (u)	-2.70 ± 4.06	-8.21 ± 3.92	PS
LPC(O-16:0)	-26.71 ± 3.66 (u, m)	-21.88 ± 3.56 (u, m)	-13.82 ± 3.89 (u, m)	-12.89 ± 5.15 (u)	-8.06 ± 5.08	-4.83 ± 4.91	Non-specific
LPC(16:1)	-12.83 ± 3.98 (u)	-13.71 ± 3.87 (u)	-3.30 ± 4.23	-9.53 ± 5.66	-10.42 ± 5.58 (m)	0.88 ± 5.39	PS
LPC(16:0)	-20.03 ± 2.63 (u)	-16.55 ± 2.56 (u, m)	-10.70 ± 2.79 (u, m)	-9.32 ± 3.72 (u)	-5.85 ± 3.67	-3.48 ± 3.55	Non-specific
LPC(18:1)	-11.86 ± 3.50 (u)	-6.68 ± 3.40	1.49 ± 3.72	-13.35 ± 5.00 (u)	-8.17 ± 4.93	-5.18 ± 4.77	
LPC(18:0)	-21.52 ± 2.82 (u, m)	-14.97 ± 2.75 (u, m)	-9.60 ± 3.01 (u, m)	-11.93 ± 4.04	-5.38 ± 3.99	-6.55 ± 3.85	Non-specific
LPC(20:4)	-15.58 ± 3.79 (u)	-5.96 ± 3.69	-1.40 ± 4.04	-14.18 ± 5.45 (u)	-4.56 ± 5.38	-9.62 ± 5.20	
LPC(20:3)	-14.11 ± 5.05 (u)	-5.91 ± 4.91	9.80 ± 5.37	-23.91 ± 7.19 (u)	-15.71 ± 7.10 (m)	-8.20 ± 6.86	PS
LPC(20:1)	-26.58 ± 4.70 (u, m)	-17.93 ± 4.57 (u)	-5.96 ± 4.99	-20.62 ± 6.64 (u, m)	-11.97 ± 6.55	-8.65 ± 6.33	PS
LPC(20:0)	-14.34 ± 5.24 (u)	-8.39 ± 5.10	-7.24 ± 5.57	-7.10 ± 7.39	-1.16 ± 7.29	-5.94 ± 7.04	
LPC(22:6)	-13.66 ± 5.06 (u)	-13.60 ± 4.92 (u)	-6.42 ± 5.38	-7.24 ± 7.23	-7.18 ± 7.13	-0.05 ± 6.89	
LPE(18:0)	-22.83 ± 4.50 (u)	-18.65 ± 4.38 (u, m)	-13.80 ± 4.78	-9.03 ± 6.36	-4.86 ± 6.27	-4.18 ± 6.06	
PC(O-34:3)	-9.66 ± 2.64 (u)	-3.22 ± 2.57	-3.21 ± 2.82	-6.46 ± 3.82	-0.01 ± 3.77	-6.45 ± 3.64	
PC(O-34:1)	-5.41 ± 1.97 (u)	-0.40 ± 1.92	3.76 ± 2.10	-9.17 ± 2.84 (u)	-4.16 ± 2.81	-5.01 ± 2.71	
PC(O-36:6)	-14.94 ± 7.53	-19.77 ± 7.33 (u)	-1.81 ± 8.00	-13.13 ± 10.64	-17.96 ± 10.50	4.83 ± 10.14	
PC(O-36:5)	-10.57 ± 2.56 (u, m)	-0.90 ± 2.48	-2.75 ± 2.72	-7.82 ± 3.69	1.85 ± 3.64	-9.67 ± 3.52 (m)	Fat content
PC(O-36:3)	-12.77 ± 3.77 (u)	-11.41 ± 3.66 (u, m)	-8.25 ± 4.01	-4.52 ± 5.42	-3.17 ± 5.34	-1.35 ± 5.16	
PC(O-38:7)	-10.52 ± 4.26 (u)	-4.61 ± 4.14	-5.77 ± 4.54	-4.75 ± 6.11	1.16 ± 6.03	-5.91 ± 5.83	
PC(O-38:4)	-10.78 ± 2.46 (u)	1.73 ± 2.39	-1.31 ± 2.62	-9.48 ± 3.56 (u)	3.04 ± 3.51 (m)	-12.51 ± 3.39 (u, m)	Fat content
PC(32:1)	11.76 ± 6.75 (m)	1.35 ± 6.56	18.15 ± 7.17 (u)	-6.39 ± 9.57	-16.80 ± 9.44	10.41 ± 9.13	
PC(34:3)	2.09 ± 4.01	3.96 ± 3.90	13.98 ± 4.26 (u)	-11.89 ± 5.71	-10.02 ± 5.64	-1.87 ± 5.45	
PC(36:3)	-0.57 ± 2.55	3.26 ± 2.48	8.96 ± 2.72 (u, m)	-9.53 ± 3.66 (u)	-5.70 ± 3.61	-3.83 ± 3.49	
PC(36:2)	-1.68 ± 2.05	3.82 ± 1.99	5.87 ± 2.18	-7.55 ± 2.93	-2.05 ± 2.89	-5.50 ± 2.80 (m)	
PC(36:1)	2.38 ± 4.41	-0.31 ± 4.29	12.73 ± 4.68 (u, m)	-10.35 ± 6.18	-13.04 ± 6.09	2.69 ± 5.89	
PC(38:7)	-6.68 ± 9.79	-15.75 ± 9.23	22.16 ± 10.38	-28.84 ± 13.67	-37.91 ± 13.48 (m)	9.07 ± 13.03	
PC(38:6)	-2.68 ± 3.43	-10.54 ± 3.33 (u)	-4.50 ± 3.65	1.83 ± 4.92	-6.04 ± 4.86	7.87 ± 4.69	
PC(40:6)	-1.31 ± 3.56	-9.58 ± 3.46 (u)	-3.31 ± 3.79	2.00 ± 5.14	-6.27 ± 5.07	8.26 ± 4.90	
SM(d18:1/15:0)	-6.49 ± 1.62 (u)	-2.85 ± 1.58	1.51 ± 1.73	-8.00 ± 2.35 (u, m)	-4.36 ± 2.32	-3.65 ± 2.24	PS-A
SM(d18:1/16:1)	-6.45 ± 1.45 (u)	-3.20 ± 1.41	0.81 ± 1.55	-7.26 ± 2.10 (u)	-4.01 ± 2.07 (m)	-3.25 ± 2.00	PS
SM(d18:1/16:0)	-7.42 ± 1.50 (u)	-4.45 ± 1.46 (u)	0.98 ± 1.60	-8.41 ± 2.16 (u, m)	-5.43 ± 2.13	-2.97 ± 2.06	PS

Table 2 continued

Lipid ^a	Effect ^b of intervention ^c versus baseline			Effect of intervention versus effect of other intervention			Assigned effect ^d
	A	B	C	A versus C	B versus C	A versus B	
SM(d18:1/17:0)	-6.78 ± 2.13 (u)	-4.23 ± 2.07	0.77 ± 2.27	-7.55 ± 3.07 (u)	-5.00 ± 3.02	-2.54 ± 2.92	PS-A
SM(d18:1/18:1)	-6.26 ± 2.19 (u)	-4.75 ± 2.13	0.65 ± 2.34	-6.91 ± 3.16 (u)	-5.40 ± 3.12	-1.51 ± 3.01	PS-A
SM(d18:1/18:0)	-6.86 ± 2.18 (u)	-5.80 ± 2.11 (u)	-0.46 ± 2.32	-6.40 ± 3.13	-5.34 ± 3.08	-1.06 ± 2.98	
SM(d18:1/20:1)	-6.05 ± 1.83 (u)	-3.93 ± 1.78	-1.96 ± 1.95	-4.09 ± 2.65	-1.97 ± 2.61	-2.12 ± 2.52	
SM(d18:1/20:0)	-6.00 ± 1.80 (u)	-2.46 ± 1.75	1.50 ± 1.92	-7.49 ± 2.60	-3.95 ± 2.57	-3.54 ± 2.48	
SM(d18:1/21:0)	-4.97 ± 1.96 (u)	-2.03 ± 1.90	2.50 ± 2.08	-7.48 ± 2.83 (u)	-4.53 ± 2.80	-2.95 ± 2.70	PS-A
SM(d18:1/22:1)	-6.08 ± 1.71 (u)	-4.08 ± 1.66	2.07 ± 1.82	-8.15 ± 2.47 (u, m)	-6.15 ± 2.43	-2.00 ± 2.35	PS-A
SM(d18:1/22:0)	-7.75 ± 2.13 (u)	-4.01 ± 2.07	1.71 ± 2.26	-9.46 ± 3.04 (u)	-5.71 ± 3.00	-3.74 ± 2.90	PS-A
SM(d18:1/23:1)	-8.20 ± 1.81 (u)	-5.41 ± 1.76 (u)	0.92 ± 1.93	-9.13 ± 2.62 (u, m)	-6.33 ± 2.59	-2.79 ± 2.50	PS-A
SM(d18:1/23:0)	-7.12 ± 2.09 (u)	-4.85 ± 2.03	2.21 ± 2.22	-9.33 ± 2.99 (u)	-7.06 ± 2.95	-2.27 ± 2.85	
SM(d18:0/24:2)	-6.92 ± 1.68 (u)	-5.52 ± 1.63 (u)	0.59 ± 1.79	-7.51 ± 2.43 (u)	-6.12 ± 2.40	-1.40 ± 2.32	
SM(d18:0/24:1)	-6.55 ± 1.81 (u)	-6.88 ± 1.76 (u)	0.76 ± 1.93	-7.31 ± 2.59 (u)	-7.63 ± 2.56 (m)	0.32 ± 2.47	PS
SM(d18:0/24:0)	-7.88 ± 2.33 (u)	-4.29 ± 2.26	2.95 ± 2.48	-10.83 ± 3.33 (u)	-7.24 ± 3.28	-3.59 ± 3.17	
SM(d18:0/25:1)	-9.73 ± 2.84 (u)	-3.71 ± 2.76	5.54 ± 3.02	-15.27 ± 4.09 (u, m)	-9.25 ± 4.03 (m)	-6.02 ± 3.90 (m)	PS
SM(d18:0/25:0)	-10.99 ± 2.78 (u)	-1.44 ± 2.71	3.50 ± 2.96	-14.49 ± 4.01 (u, m)	-4.93 ± 3.95	-9.55 ± 3.82 (u, m)	PS-A + fat content
TG(54:5)	-3.91 ± 6.89	0.62 ± 6.70	22.68 ± 7.32 (u)	-26.59 ± 9.81 (u)	-22.06 ± 9.67	-4.53 ± 9.35	

CE cholesteryl ester, *LPC* lysophosphatidylcholine, *LPE* lysophosphatidylethanolamine, *PC* phosphatidylcholine, *SM* sphingomyelin, *TG* triacylglycerol

^a Only lipids with statistically significant effect ($P < 0.05$) in univariate (u) and/or multivariate (m) data analysis are presented here as described in [Materials and methods](#) section

^b Effects are mean differences in levels of a lipid expressed in % compared to baseline levels ± SD

^c Interventions: A Plant sterol PS-enriched yoghurt drink with 0.1% dairy fat and 2.2% total fat, B PS-enriched yoghurt drink with 1.5% dairy fat and 3.3% total fat, C Placebo yoghurt drink with 1.5% dairy fat

^d Assigned effects: PS plant sterol effect, PS-A plant sterol effect specific for drink A, fat content effect, non-specific non-specific effect

and LPC(18:0). A small group of LPCs including LPC(O-16:1), LPC(18:1), LPC(20:4), LPC(20:3) and LPC(20:1) was differently affected by at least one of the plant-sterol interventions than by the placebo intervention.

In the group of phosphatidylcholines, levels of 15 out of 31 metabolites were increased or decreased after the intervention period when compared to baseline. The mean differences when compared to baseline ranged between -20% (PC(O-36:6) after intervention B) and $+22\%$ (PC(38:7) after placebo intervention). An increase in concentrations of some PCs was mainly observed after the placebo intervention and was statistically significant for PC(32:1), PC(34:3), PC(36:3) and PC(36:1). When effects of plant sterol treatments were compared with effects of the placebo, levels of only four metabolites were affected differently (PC(O-34:1), PC(O-38:4), PC(36:3), PC(38:7)).

In the group of sphingomyelins, levels of 18 out of 20 metabolites were significantly decreased but their decrease was usually not more than 10% (except SM(d18:1/25:0)). When effects of PS drinks were compared with effects of placebo, most SM changes observed after PS drink A were statistically relevant, while after PS drink B changes in levels of only three SMs were statistically relevant (i.e. SM(d18:1/16:1), SM(d18:0/24:1), SM(d18:0/25:1)).

All interventions had a limited effect on levels of triacylglycerols (only 1 out of 23 metabolites is affected). Only the placebo intervention showed significantly increased levels of TG(54:5) (by *ca.* 23%) when compared to baseline and to the effect of PS intervention A.

3.2 The most influential lipid metabolites

In this study metabolites which level changes were assessed as statistically significant during univariate (first data analysis approach) as well as during multivariate analysis (second or third data analysis approach) were selected to be the most influential metabolites and used for biological interpretation. They were separated into three groups to establish: (1) the influence of plant sterols—lipids that were differently affected by PS-enriched drinks A and B than by the placebo drink C, (2) the influence of fat content of the PS-enriched drink—lipids that were differently affected by the 1.5% dairy fat PS-enriched drink B and 0.1% dairy fat drink A, (3) non-specific effects of all intervention—lipids that were significantly affected by all three drinks: A, B and C when compared to the baseline levels, but for which no statistically significant difference was found when the effects of different drinks were compared with each other. Some metabolites belonged to more than one group e.g. SM(d18:0/25:0). In Table 2 lipids are assigned to one of three main groups of effects.

To the set of lipids affected by PS belonged several CEs, LPCs, and SMs. Within this set a subset could be

distinguished which was significantly affected only after the 0.1% dairy fat PS drink A but not significantly after 1.5% dairy fat PS drink B intake. To this subset of lipids belong e.g. CE(18:2) and SM (d18:1/15:0). The set of lipids mostly affected by the fat content of the drinks included e.g.: PC(O-36:5), PC(O-38:4) and SM(d18:0/25:0). The set of lipids affected by all drinks irrespective of their PS and fat content contained three LPCs: LPC(O-16:0), LPC (16:0) and LPC(18:0).

3.3 Comparison to lipoprotein profiles

In the previous study, statistically significant decreases in LDL-C and TC concentrations (*ca.* $9.3\text{--}9.5\%$ and $6.5\text{--}7\%$, respectively) were found for both PS-enriched yoghurt drinks (A and B) when compared to placebo drink C (Doornboos et al. 2005). The current lipidomics analysis showed that also the serum levels of many lipid metabolites decreased after 4-weeks of yoghurt drink A and B when compared to placebo drink.

Associations between the effects of PS enriched yoghurt drinks on serum lipidomic profiles and on lipoprotein parameters were investigated by correlation analysis and PLS regression analysis. An interesting strong positive correlation was observed between changes of several SMs and LDL-C and TC concentrations particularly for the intervention group A (see Supplementary Fig. 3). The group of SMs was clearly affected by the PS-enriched drink A and the effect size was similar to those of LDL-C and TC (sphingomyelins: -7 to -9% with respect to placebo intervention). Results of the PLS regression analysis in which the differences in serum levels of only 20 SMs were regressed against differences in LDL-C and TC concentrations are shown in Table 3. The relationship between changes in SMs and LDL-C and TC is the strongest within the intervention group A which has the strongest effects on lipidomics profiles.

4 Discussion

In the current study lipidomics profiles were analyzed to explore lipid biochemical changes occurring during a parallel group 4-week nutritional intervention. These changes were related to plant sterol effects, fat content effects and non-specific effects of tested yoghurt drinks. A comprehensive data analysis strategy was applied to study these different effects. It included comparisons of drink effects in respect to effect of other drink and in respect to baseline as well as analysis on both univariate and multivariate level.

It was shown that two the tested PS-enriched yoghurt drinks, which earlier showed a similar reduction in TC and LDL-C, have significant but different effects on the serum

Table 3 Performance parameters for the partial least squares (PLS) calibration models for the association between changes in LDL-C and TC levels and changes in SMs profiles of groups A, B and C

Models/performance parameters	SM profile/LDL-C models			SM profile/TC models		
	Group A ^a	Group B ^b	Group C ^c	Group A ^a	Group B ^b	Group C ^c
RMSEP ^d	0.42	0.66	0.85	0.49	0.75	0.88
<i>r</i> ^e	0.84	0.46	0.56	0.88	0.49	-0.63

^a Group of subjects treated with plant sterol (PS)-enriched yoghurt drink with 0.1% dairy fat and 2.2% total fat

^b Group of subjects treated with PS-enriched yoghurt drink with 1.5% dairy fat and 3.3% total fat

^c Group of subjects treated with placebo yoghurt drink with 1.5% dairy fat

^d Root mean squared error of prediction

^e Pearson correlation coefficient calculated between predicted values versus original values

lipidome. Particularly the 0.1% dairy fat, PS-enriched yoghurt drink had a strong effect on the lipidome, as is shown in Table 2. The main common effect of both PS-drinks (plant sterol effect) was related to changes in phospholipids, mainly SMs, LPCs and cholesteryl esters. This in agreement with results of a recent study on therapeutic and adverse effects of cholesterol-lowering drugs like statins: rosuvastatin and atorvastatin on lipidome (Bergheanu et al. 2008). There changes in LDL-C/HDL-C ratios were also found to be correlated to a number of SMs and PCs. Also Kaddurah-Daouk et al. (2010) recently reported that changes in concentrations of cholesteryl ester and phospholipid metabolites correlated with LDL-cholesterol response to simvastatin. In our study changes in sphingomyelins showed the best correlation with LDL-cholesterol response while they were not included in the simvastatin lipidomics study.

A significant reduction of serum levels of several SMs, well correlated with the reduction in LDL-C is the most consistent effect of PS observed in our study. SM and cholesterol levels are coordinately regulated in in vitro model systems and in human disorders (Ridgway 2000). SMs are typically co-localized to cholesterol in membranes and lipoprotein surface (Ramstedt and Slotte 2006). Moreover, elevated plasma levels of SMs also have been shown to be a risk factor for coronary artery disease (CAD) (Jiang et al. 2000; Schlitt and Blankenberg 2006). In CAD patients, plasma SM levels showed strong correlation with apoB and triglyceride-rich lipoproteins (Nilsson and Duan 2006; Schlitt and Blankenberg 2006). Plasma SM levels have been associated with the formation of atherogenic lipoprotein aggregates, which is the initial step of atherosclerosis. A recent study by Stegmann showed that certain SM species were relatively more enriched in atherosclerotic plaques than in corresponding plasma samples (Stegmann et al. 2011). Additionally, SMs present in cell membranes and lipoproteins are the source for a variety of other sphingolipids. These include signaling lipids like

ceramides and sphingosine-1-phosphate, which likely play important roles in inflammation processes (Nixon 2009).

Plasma levels of several LPC's were also reduced following consumption of PS-enriched yoghurt drinks, particularly LPC(16:1), LPC(20:1), LPC(O-16:1). LPC is a major phospholipid component of oxidized LDL (Ox-LDL) and is believed to be critical for the atherogenic activity of Ox-LDL (Schmitz and Ruebsaamen 2010). On the other hand also cholesteryl esters (CE(20:4) and CE(18:2)) were found to be lowered upon intake of PS-enriched yoghurt drinks. The lipid core of LDL consists primarily of CEs, so it is not surprising that along with PS-induced LDL-C lowering, plasma levels of CEs are reduced. Cholesteryl esters with polyunsaturated fatty acids (e.g. cholesteryl arachidonate (CE(20:4))) were highly enriched in atherosclerotic plaques as compared to control arteries with cholesteryl linoleate (CE(18:2)) being the main CE species in plaques (Stegmann et al. 2011). Arachidonic acid and linoleic acid are well-known precursors to a range of pro-inflammatory eicosanoids. Thus, reduced serum levels of these lipids may also suggest improved inflammatory status, though clearly more mechanistic understanding of this area is needed.

Human data in the literature on effects of PS intake on inflammation markers are scarce and conflicting, but some published in vitro studies support our current findings suggesting that PS may have anti-inflammatory activity (Brüll et al. 2009). Also, (Lankinen et al. 2009) showed recently that 8-week intake of fatty fish altered the lipidomic profile, and linked the reduction of lyso-PCs and ceramides to anti-inflammatory effects of *n*-3 fatty acids. Another lipidomic study showed that 3-week intake of probiotics in healthy adults (Kekkonen et al. 2008) reduced levels of lyso-PCs, PCs, and SMs, while particularly cytokine IL-6 levels were associated with LPC(20:4).

Our applied lipidomics approach was able to find also non-specific effects of intervention period (LPC(O-16:0), LPC(16:0) and LPC(18:0)) as well as fat content effects

(especially in changes of phospholipids: PC(O-36:5), PC(O-38:4) and SM(18:0/25:0)) observed between the groups that consumed the 0.1% dairy fat and the 1.5% dairy fat PS-enriched yoghurt drinks. Usually intake of the higher fat yoghurt drink (B) led to much fewer significant effects on the lipidome than did the lower fat drink (A). It was not expected that the fat content would significantly affect the impact of PS intake on serum lipids, since all subjects consumed the product together with a lunch meal. This meal contained a sufficient amount of fat to stimulate bile flow and allow formation of dietary mixed micelles in the intestine (Doornboos et al. 2005). This was important since the main hypothesis on the mechanism of action of PS is that they compete with cholesterol and displace it from dietary mixed micelles, causing less cholesterol to be transported through the intestinal brush border membrane (de Jong et al. 2003; Trautwein et al. 2003). The different effects of the two PS-enriched drinks A and B on the serum lipidome as observed here are unexpected and currently difficult to explain, but do not indicate different efficacy of the two drinks. It is likely that the comprehensive serum lipidomics is able to pick up blood lipid effects much more sensitively than a standard lipoprotein analysis, and e.g. may detect differences in genetic make-up and/or background diet between (sub-) populations in the two intervention groups. At a more detailed level, it is possible that the lipoprotein particle size distribution within the three treatment group populations were slightly different. A more detailed analysis of the lipoprotein subclass/particle size distribution would be required to indicate whether differences in lipidome effects between the PS-intervention groups may be associated with effects on lipoprotein particle size distribution that are not picked up with the standard clinical analysis.

5 Conclusion remarks

This is the first study which applies lipidomic profiling to study effects of a PS intervention on lipid metabolism. The effect of PS on lipidomic profiles was characterized and related to the changes in standard lipoprotein profiles (LDL-C, HDL-C, TC and TAG) in healthy mildly hypercholesterolaemic subjects. We here demonstrate a novel and comprehensive data analysis strategy to assess and compare effects of three different treatments in a parallel group study. Combining information of univariate and multivariate data analysis approaches gave more insight in the subtle effects of a nutrition intervention, and helped to distinguish PS-related effects from product matrix (i.e. fat content) or non-specific effects of all treatments.

Consumption of the PS-enriched 0.1% dairy fat yoghurt drink had the most significant impact on the serum

lipidome, including reductions in several SMs, LPCs and cholesteryl-arachidonide, which may suggest reduced inflammation and atherogenic potential. Further studies are needed to explain the fewer effects of the 1.5% dairy fat yoghurt drink, despite similar LDL-C lowering effects.

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